TOXIC AND ANTAGONISTIC EFFECTS OF SALTS AS RELATED TO AMMONIFICATION BY *BACILLUS SUBTILIS*

CHAS. B. LIPMAN

(WITH FIVE FIGURES)

In the science of bacteriology, especially in that of soil bacteriology, our work has thus far only taught us enough to give added zest to investigation and the most important and interesting results are still forthcoming. RAMANN (23) well expresses it when he says, "In spite of numerous and important investigations we are still but in the first stages of our researches on bacteria and only at the very starting-point in our knowledge of soil bacteria." In his haste to classify bacteria, to show their direct relation to many diseases, to apply his knowledge of them to the practical side of the dairy industry, and finally, to measure the net results of the activities of bacteria in the soil, the bacteriologist has left almost untouched one of the most important phases of the science of bacteriology, namely, the physiology of bacteria, which is of great scientific interest and practical importance. Especially is this true of the physiology of soil bacteria, which remains as yet a closed book, and since the writer is devoting his time to researches on soil bacteria in particular, it was thought best at first to experiment with some of these organisms. It may also be added that in California, with its thousands of acres of waste alkali land, and in similar regions elsewhere this study will undoubtedly prove to be of the greatest practical significance, especially when we have learned to coordinate the results obtained in similar investigations on the higher plants with those derived from researches on soil bacteria.

Since ammonification is the first great step in the transformation and simplification of the organic soil nitrogen, it was thought best to study the effect of various salt solutions on pure cultures of ammonifiers first. The work was carried out along the same general lines as to the preparation of solutions as the experiments of LOEB on the effects of salt solutions on various forms of animal life, and the subse-

quent experiments of OSTERHOUT on the higher plants. It was indeed the insight of the latter investigator which led him to conclude some time ago that a proper understanding of physiologically balanced solutions in relation to plants and to soil bacteria would render the control and successful cultivation of alkali lands a much simpler matter than it has been, and this will undoubtedly prove true in the near future.

In the selection of an ammonifier which could be used uniformly throughout all the experiments, the writer was guided by the work of MARCHAL (15), to whom indeed we owe what little knowledge we have of the physiology of ammonifiers. Among the best ammonifiers found by that investigator were *B. mycoides*, which changed 46 per cent. of nitrogen into ammonia in a given time, *Proteus vulgaris* (36 per cent.), *Sarcina lutea* (27 per cent.), and *B. subtilis* (19 per cent). Since the last form is easily isolated and cultivated and is a strong ammonifier, it was decided to use it in the following experiments. It is more than probable that the same relative results will be obtained with any ammonifier; this, however, will be tested in other experiments now contemplated by the writer. The pure culture of *B. subtilis* employed for inoculation through all the series of experiments was obtained from soil from Auburn in the foothill fruit region of California.

The salts tested were the chlorids of sodium, potassium, calcium, and magnesium. Only chemically pure salts were used, after submitting them to a flame test. Molecular or bimolecular stock solutions in distilled water were made, from which the requisite amounts were taken for the various concentrations. Witte's peptone was the nitrogenous substance used for the ammonification, of which 1 per cent. solutions were employed in the tests for the single salts and 0.75 per cent. in the binary solutions. The method of inoculation employed was as follows: Inoculation is made from peptone agar slope of *B. subtilis* into a sterile 100^{cc} portion of 1 per cent. peptone in a 250^{cc} Erlenmeyer flask. This is incubated for forty-eight hours at 28° C., at the end of which time the membrane that forms on the surface of the culture is precipitated by slight shaking, and then by tilting the flask to one side and carefully setting it down again, the liquid covering part of the bottom of the flask remains free from membranous

material and is homogeneous in character. Of this homogeneous liquid 1^{cc} was drawn off with a sterile pipette for inoculation into each flask to be tested, the greatest caution being used to prevent any particle of membrane from entering the pipette as the liquid was drawn up. This was the most satisfactory method of inoculation of several tested and yields concordant results in the duplicate series. All the solutions employed were made practically neutral. The incubation was carried out in a thermostat at a temperature which varied between 28° and 29° C. The incubation period was two days in the case of the single salt solutions, and two and one-half days for the binary solutions. The amount of ammonia formed, which was used as a criterion for establishing the efficiency of *B. subtilis* in the various solutions, was determined as follows: At the end of the incubation period the culture solutions were transferred to flat-bottomed Jena distillation flasks, diluted to $300-350^{cc}$, an excess of magnesium oxid added, and distilled. The amount of ammonia in the distillate was titrated against standard acid, cochineal being used as the indicator. Sterile blanks were run on all determinations, each of which was made in duplicate, and the tables given below represent averages of at least three sets of such duplicates and in some instances of five and six sets of duplicates.

Experiments with single salts

In determining the salts to be tested the writer was guided by the alkali and alkaline earth constituents of soils. The sodium, potassium, calcium, and magnesium salts are important factors in plant nutrition and are always present in soils; in some cases, indeed, one or more of them may be present in such excess as to inhibit plant growth materially and in some instances completely. Such soils we find in California and other states under the common appellation of "alkali lands." It was decided, therefore, to test the salts of the alkalies and alkaline earths above mentioned, to determine the degree of toxicity of each for the bacteria experimented upon. Since from similar work on animals and plants, the anion of salts was found to have comparatively little effect, a chlorid of each metal was employed for the sake of uniformity.

SERIES I. SODIUM CHLORID

There were prepared sixteen Erlenmeyer flasks (125^{cc} capacity), each containing 50^{cc} of solution, made up as follows: The first flask contained 50^{cc} of 1 per cent. peptone solution in distilled water; the second contained a 0.1 *m* solution of NaCl and 1 per cent. peptone; the third flask contained 0.2 *m* NaCl and 1 per cent. peptone; and so on, the concentration of NaCl increasing by 0.1 *m* in each succeeding flask up to the sixteenth, which contained 1.5 *m* NaCl and 1 per cent. peptone. All the flasks were plugged with cotton, sterilized in the autoclave at a pressure of 1.25 atmospheres, and when cool were inoculated from a culture of *B. subtilis* in the manner above described. After two days' incubation at 28° to 29° C., the ammonia formed in the peptone solutions was distilled off and determined as above explained. Table I shows the results.

TABLE I

Numbers represent tenths *m* NaCl solution	Milligrams of N formed as NH_3
0	5.60
1	6.79
2	4.13
3	2.80
4	2.59
5	2.45
6	2.38
7	2.10
8	1.89
9	1.54
10	1.33
11	1.05
12	0.91
13	0.28
14	0.28
15	0.28

Plotting a curve by laying off the concentrations as abscissae and the milligrams of N (as NH_3) as ordinates (*fig. 1*), we note the following: NaCl up to a concentration of 0.1 *m* stimulates *B. subtilis* as an ammonifier, but beyond that concentration it becomes gradually more and more toxic, and at 1.3 *m* we find scarcely any ammonification. It is evident, also, that NaCl is not nearly as toxic for *B. subtilis* as it has been found by LOEB (**8**, p. 412)[1] and OSTWALD (**21**) to be for animals, and as OSTERHOUT (**17**) and MAGOWAN (**14**) have found it to be for plants. LOEB found, for instance, that the formation of embryos in the eggs of Fundulus was rendered impossible in a 0.625 *m* solution of NaCl, whereas in a 0.7 *m* solution of the same salt *B. subtilis* makes a fairly good growth and forms a considerable amount of ammonia. As an extreme example of the toxicity of NaCl, OSTERHOUT (**18**) found that zoospores of *Vaucheria sessilis* placed in a 0.0937 *m* solution of NaCl usually died within a few min-

[1] See also **5** and literature there cited.

utes, and further, that even at a dilution of 0.0001 *m* NaCl proved poisonous to the young plants. The results above given serve, however, to confirm again the general principle, formulated by LOEB, of the toxicity of NaCl alone for living organisms.

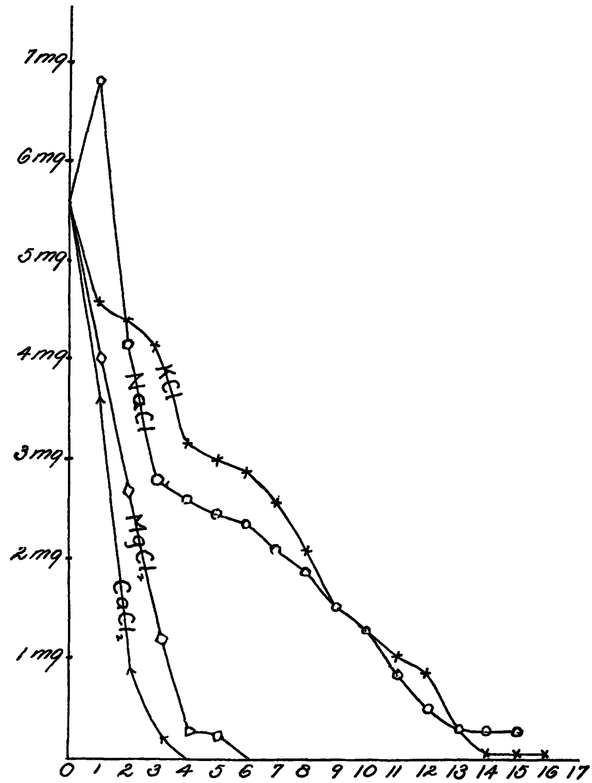

FIG. 1.—Toxicity curves of KCl, NaCl, $MgCl_2$, and $CaCl_2$. The ordinates represent milligrams of ammonia nitrogen. The ordinate at 0 represents the amount of ammonia nitrogen formed in a blank culture (1 per cent. peptone in distilled water). The abscissae represent concentrations in tenths molecular.

SERIES II. CALCIUM CHLORID

Here the experiment was carried out in the same general way as in series I, $CaCl_2$ being substituted for NaCl, and, owing to the extreme toxicity of the former, solutions ranging from 0.1 *m* to 0.6 *m* only were prepared. The ammonia was determined as above and the results are shown in table II.

Here again (see *fig. 1*) we find agreement between the toxic action of $CaCl_2$ on bacteria with that of the same salt and other calcium salts on animals as shown by LOEB (**8**, p. 425) in the case of Fundulus, and by OSTWALD (**21**) in his work on the freshwater Gammarus. It is well to note here also that an examination of the four curves (*fig. 1*) for the salts employed reveals the fact that $CaCl_2$ is easily the most toxic of all for *B. subtilis*. This fact is of especial interest because of its wide disagreement with the facts obtained by experiments on plants with $CaCl_2$, in which MAGOWAN (**14**), for example, found it to be the least toxic of the four salts for wheat (variety Early Genesee). In this respect therefore, if *B. subtilis* may be considered representative, bacteria exhibit the physiological characteristics more typical of animals than of plants, with which they are now classed. We see above, that even at a concentration of 0.3 *m* $CaCl_2$ the formation of ammonia by *B. subtilis* is inhibited.

TABLE II

Numbers represent tenths *m* $CaCl_2$ solution	Milligrams of N formed as NH_3
0	5.60
1	3.15
2	0.91
3	0.21
4	0.00
5	0.00
6	0.00

SERIES III. POTASSIUM CHLORID

The experiment was arranged as those preceding it and the results are shown in table III.

On examining the curve (*fig. 1*) obtained from these results we see at once the strong resemblance of it to that obtained with the solution of NaCl, and although at the concentrations employed KCl exhibits no stimulating effect, it may show it at some concentration lower than 0.1 *m*. This general agreement of the chlorids of K and Na has been found to be even more striking by MAGOWAN (**14**) in a series of experiments on wheat. Here, therefore, *B. subtilis* exhibits physiological characteristics akin to those of the higher plants and differing widely from those of animals, as shown, for

TABLE III

Numbers represent tenths *m* KCl solution	Milligrams of N formed as NH_3
0	5.60
1	4.55
2	4.41
3	4.13
4	3.15
5	3.01
6	2.87
7	2.59
8	2.10
9	1.54
10	1.33
11	0.84
12	0.49
13	0.28
14	0.07
15	0.07
16	0.07

example, by the work of OSTWALD (21) in which KCl showed the most extreme toxicity of all the salts tested.

It is also worthy of mention here, as shown above, that the KCl curve declines more gradually than the sodium curve beyond the concentrations of 0.1 *m*.

SERIES IV. MAGNESIUM CHLORID

In the curve drawn on the basis of table IV (*fig. 1*) we notice a parallel to the toxicity curve for $CaCl_2$, except that $MgCl_2$ is not nearly as toxic for *B. subtilis* as the former. While a 0.3 *m* solution of $CaCl_2$ totally inhibits the ammonifying activity of *B. subtilis*, the latter will make a fair growth in a solution of $MgCl_2$ of like concentration and form an appreciable amount of ammonia. Here again, we find agreement between the behavior of *B. subtilis* and Fundulus in solutions of $MgCl_2$, as LOEB (7, p. 411) found that in an *m*/2 solution of $MgCl_2$ no embryo develops in the eggs of Fundulus, but that the same degree of toxicity is reached in an *m*/8 solution of $Ca(NO_3)_2$, thus showing the magnesium salt to be less toxic than the calcium salt. On the other hand, as shown by MAGOWAN (14), $MgCl_2$ is far the most toxic of the four chlorids employed in experiments on wheat; and in this respect the behavior of *B. subtilis* resembles that of Fundulus rather than that of the higher plants.

TABLE IV

Numbers represent tenths *m* $MgCl_2$ solution	Milligrams of N formed as NH_3
0	5.60
1	4.00
2	2.70
3	1.20
4	0.28
5	0.28
6	0.00

Experiments with binary solutions

The fact that the results obtained were in such striking general agreement with those of the investigators above mentioned on animals and higher plants was sufficient stimulus for further inquiry into the biochemistry of ammonification. It was deemed of interest, therefore, to see if antagonism between salts holds as well for bacteria as it does for the higher forms of life, with the end in view of ascertaining whether balanced solutions are necessary for bacteria, and whether solutions balanced for the other forms of life investigated will prove the same for bacteria. The remainder of this paper will deal with the antagonistic effects of one salt on another in binary solutions, while

the establishment of a balanced solution, with other work now contemplated by the writer, will form the subject of another article.

SERIES V. POTASSIUM CHLORID *vs.* CALCIUM CHLORID

In these experiments the technique was somewhat different from the foregoing. All the salts were used at a concentration of 0.35 *m*[2] and mixed with each other in various proportions in Erlenmeyer flasks of 250^{cc} capacity. Two or three liters each of KCl and $CaCl_2$ solution of the concentration above noted were made up to contain 0.75 per cent. peptone.[3] In each flask of the first half of the series were placed 100^{cc} of the KCl-peptone solution. To no. 1 nothing was added; to the others up to no. 6 there were added respectively 5, 10, 25, 50, and 100^{cc} of the $CaCl_2$-peptone solution. Then beginning at the other end of the series each flask received 100^{cc} of the $CaCl_2$-peptone solution. Flask no. 11 contained this alone; to the preceding ones in order were added respectively 5, 10, 25, 50, and 100^{cc} KCl-peptone solution, the two halves of the series meeting in no. 6, as shown in the curve (*fig. 2*) and table V, at the combination of 100^{cc} of each solution. In order to keep the volume the same in all cultures they were thoroughly mixed, and enough solution drawn off with the pipette, where additions were made, to make all the culture

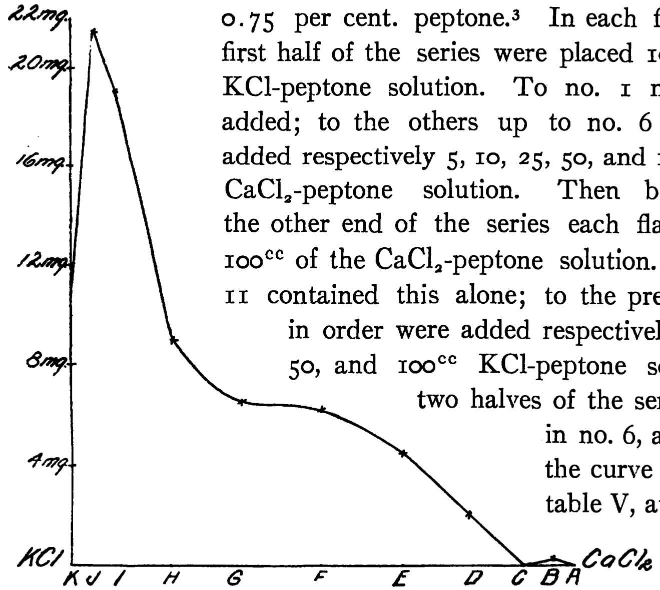

FIG. 2.—Antagonism curve, KCl *vs.* $CaCl_2$. The ordinate at K represents the ammonia nitrogen in milligrams formed in a pure KCl solution. The ordinate at A represents the amount of ammonia nitrogen formed in a pure $CaCl_2$ solution, and the ordinates at the intermediate points represent the amounts formed in various combinations of the two salts as indicated by the corresponding letters in table V.

[2] This concentration was chosen because it was about the concentration of NaCl in sea water of San Francisco Bay.

[3] This concentration of peptone was used in all the experiments with binary solutions.

solutions have a uniform bulk of 100^{cc}, thus avoiding any differences in the supply of oxygen to the bacteria by having an equal surface of liquid exposed to the air in each flask.

As in the case of the single salt solutions, duplicates were run on all the cultures, and also sterile controls, so as to allow of a determination of the ammonia actually formed by the bacteria. The solutions were all sterilized in the autoclave at 1.25 atmospheres, inoculated as above, and incubated for two and a half days at 28° to 29° C., after which they were distilled, as in the case of the single salt cultures, and the ammonia determined. The results obtained follow, with the curve plotted from them in accordance with the arrange-

TABLE V

ALL QUANTITIES GIVEN REFER TO CUBIC CENTIMETERS OF 0.35 *m* SOLUTIONS

Culture solution	Corresponding points on curve	Milligrams of N formed as NH_3
100 KCl	K	10.92
100 KCl, 5 $CaCl_2$	J	21.46
100 KCl, 10 $CaCl_2$	I	18.83
100 KCl, 25 $CaCl_2$	H	9.00
100 KCl, 50 $CaCl_2$	G	6.44
100 KCl, 100 $CaCl_2$	F	6.30
50 KCl, 100 $CaCl_2$	E	4.55
25 KCl, 100 $CaCl_2$	D	0.25
10 KCl, 100 $CaCl_2$	C	0.14
5 KCl, 100 $CaCl_2$	B	0.25
100 $CaCl_2$	A	0.00

ment employed by OSTERHOUT (19). The letters along the axis of abscissas represent a given combination of the two salts as indicated in the table, and the ammonia formed is laid off on the axis of ordinates in numbers representing milligrams.

We see at a glance (*fig. 2*) that there is a strong antagonism

between $CaCl_2$ and KCl. OSTERHOUT (**20**) obtained a similar curve for the antagonism between the same two salts in his experiments on wheat. It is particularly interesting to note that both for wheat and for bacteria the maximum point of the curve is at the combination of 100^{cc} KCl solution and 5^{cc} $CaCl_2$ solution, notwithstanding the wide difference between the materials employed in the two cases, and further despite the fact that the Ca ions are most toxic for *B. subtilis* and least toxic for the wheat.

Again we find the strong antagonism above obtained is in striking accord with the results of LOEB in similar experiments on animals, both as to development (**7**) and as to muscular contraction (**6**).

SERIES VI. SODIUM CHLORID *vs.* MAGNESIUM CHLORID

In this series the experiment was arranged and carried out in the same manner as the one preceding, except that the salts used were different. The ammonia formed at the end of the period of incubation was determined with the following results:

TABLE VI

ALL QUANTITIES GIVEN REFER TO CUBIC CENTIMETERS OF 0.35 *m* SOLUTIONS

Culture solution	Corresponding points on curve	Milligrams of N formed as NH_3
100 NaCl	K	22.36
100 NaCl, 5 $MgCl_2$	J	23.75
100 NaCl, 10 $MgCl_2$	I	28.67
100 NaCl, 25 $MgCl_2$	H	24.56
100 NaCl, 50 $MgCl_2$	G	18.67
100 NaCl, 100 $MgCl_2$	F	10.49
50 NaCl, 100 $MgCl_2$	E	4.84
25 NaCl, 100 $MgCl_2$	D	3.80
10 NaCl, 100 $MgCl_2$	C	3.54
5 NaCl, 100 $MgCl_2$	B	3.36
100 $MgCl_2$	A	2.13

In the curve plotted from table VI (*fig. 3*), we note again a strong antagonism between the two salts tested, though it is not as marked as in the last series. The curve proves to be more regular than the last, probably owing to the fact that there was practically no variation in the temperature throughout the period of incubation. As LIP-

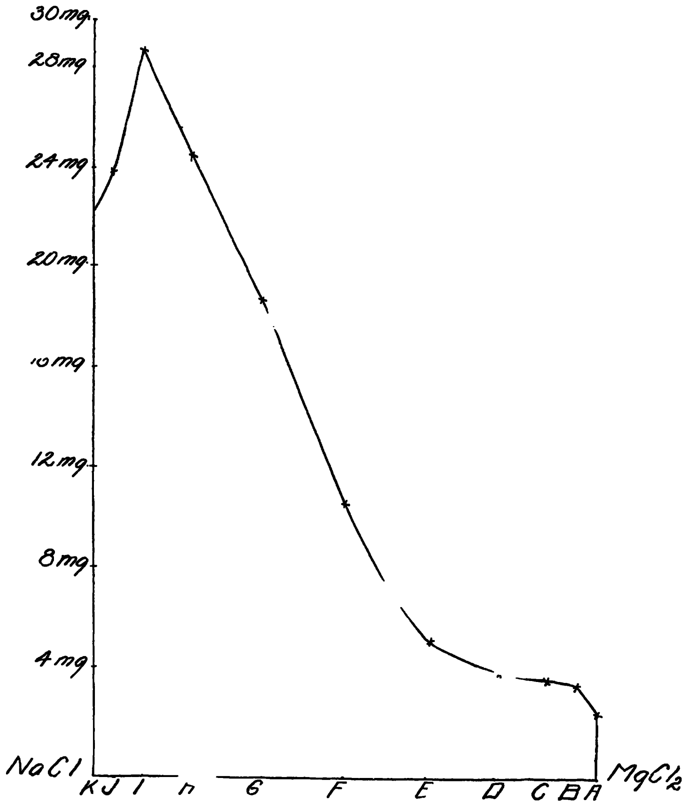

FIG. 3.—Antagonism curve, NaCl *vs.* $MgCl_2$. The ordinate at K represents the ammonia nitrogen in milligrams formed in a pure NaCl solution. The ordinate at A represents the amount of ammonia nitrogen formed in a pure $MgCl_2$ solution, and the ordinates at the intermediate points represent the amounts formed in various combinations of the two salts as indicated by the corresponding letters in table VI.

MAN (4) has demonstrated, a constant temperature and equal periods of incubation are essential factors in quantitative work in ammonification, if results are to be considered comparable.

It is a significant fact that here again the maximum point on the curve nearly coincides with that of a similar curve obtained by OSTERHOUT (19) in his experiments with the same salts on root development in wheat, and exactly coincides in the case of a fungus (*Botrytis cinerea*); and though OSTERHOUT employed such widely varying concentrations as 0.12 *m* in the case of the wheat and 1.5 *m* in Botrytis, the maximum development was reached in a mixture of 10^{cc} of the $MgCl_2$ solution (or 7.5^{cc} for wheat) and 100^{cc} of the NaCl solution, just as was the case in ammonification by *B. subtilis*.

In his experiments on the eggs of Fundulus and the sea-urchin (Arbacia), LOEB (10) found that in a mixture of 98^{cc} $5n/8$ NaCl and 2^{cc} $10n/8$ $MgCl_2$ all the eggs of Fundulus form embryos, whereas in pure NaCl or $MgCl_2$ solutions alone no embryos would form, and even in a mixture of equal parts of the above-mentioned solutions 75 per cent. of the eggs formed embryos. On the other hand, OSTWALD (21) found in his work on the freshwater Gammarus that, so far from exercising an antagonistic effect on each other, the combination of Mg and Na chlorids proved more poisonous than either alone.

SERIES VII. MAGNESIUM CHLORID *vs.* CALCIUM CHLORID

The arrangement of the experiment and the ammonia determinations were carried out in a manner similar to that employed in the two preceding series, two bivalent salts being tested this time. The results were as shown in table VII, p. 117.

By an examination of the curve drawn on the basis of table VII (*fig. 4*) we are confronted by the very striking instance of lack of antagonism between the two salts. On the contrary, there is a constant increase of the toxic properties of each when the other is added to it in increasing amounts. In this exceptional behavior, so far as the writer can ascertain, *B. subtilis* (and probably all the ammonifiers) stand alone, when their physiological efficiency in such salt mixtures is compared with that of the higher plants and animals. No instance of such behavior on the part of any member of the latter two groups of organisms has come to my notice in reviewing the results of similar researches.

An antagonism between $CaCl_2$ and $MgCl_2$, though slight, was

found to be none the less definite by LOEB (6) in experiments which showed that sea-urchin blastulae and gastrulae would swim about

TABLE VII

ALL QUANTITIES GIVEN REFER TO CUBIC CENTIMETERS OF 0.35 *m* SOLUTIONS

Culture solution	Corresponding points on curve	Milligrams of N formed as NH_3
100 $MgCl_2$	K	3.08
100 $MgCl_2$, 5 $CaCl_2$	J	2.59
100 $MgCl_2$, 10 $CaCl_2$	I	1.68
100 $MgCl_2$, 25 $CaCl_2$	H	.98
100 $MgCl_2$, 50 $CaCl_2$	G	.21
100 $MgCl_2$, 100 $CaCl_2$	F	.07
50 $MgCl_2$, 100 $CaCl_2$	E	.00
25 $MgCl_2$, 100 $CaCl_2$	D	.00
10 $MgCl_2$, 100 $CaCl_2$	C	.00
5 $MgCl_2$, 100 $CaCl_2$	B	.00
100 $CaCl_2$	A	.49

in a mixture of the salts above mentioned for forty-eight hours, while each salt by itself would immediately prove poisonous at the concen-

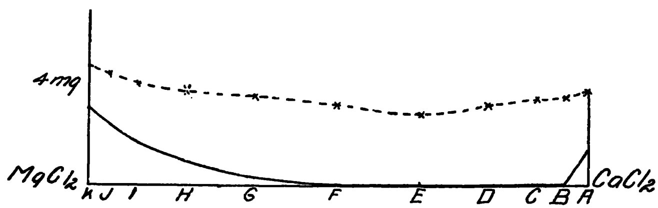

FIG. 4.—Non-antagonism curves, $CaCl_2$ *vs.* $MgCl_2$. The ordinates at K represent the ammonia nitrogen in milligrams formed in a pure $MgCl_2$ solution. The ordinate at A represents the amount of ammonia nitrogen formed in a pure $CaCl_2$ solution, and the ordinates at the intermediate points represent the amounts formed in various combinations of the two salts as indicated by the corresponding letters in tables VII (unbroken line) and VIII (dotted line).

tration employed in the combination. Another interesting case in point may be noted in the experiments of the same investigator on Polyorchis (**11**), a jelly-fish of San Francisco Bay. In a solution of 50^{cc} NaCl + 6^{cc} $MgCl_2$ + 1^{cc} $CaCl_2$, the rhythmical contractions of the margin go on normally, but with a slight increase of $CaCl_2$ the contractions are inhibited, and when 5^{cc} of a $3m/8$ solution of $CaCl_2$ are added, they are completely suppressed. On the other hand, when the margin of the fish, containing the sense organs and the central nervous system, is cut off, $CaCl_2$ exercises a stimulating action on the isolated center of the fish, and contractions will go on normally; but when $MgCl_2$ is added to the solution, in the ratio of 4 parts $MgCl_2$ to 1 part $CaCl_2$, the stimulating action of the $CaCl_2$ is suppressed and contractions cease. In both cases, therefore, there is evidence of a definite antagonism between Ca and Mg. Likewise, LILLIE (**3**) proved the existence of antagonism between the two salts, when he found that the ciliary activity of the larvae of Arenicola would go on normally for some time in a mixture of approximately 4 parts $MgCl_2$ to 1 part $CaCl_2$, whereas it would immediately cease if either of the salts at the same concentration was present alone.

Again, we find the well-known researches of LOEW and his pupils (**12, 13**), and later the researches of KEARNEY and CAMERON (**1**), which show in the higher plants the strong antagonism between calcium and magnesium. The last-named investigators found, in their experiments with the white lupin (*Lupinus albus*) and with alfalfa (*Medicago sativa*), that when $CaCl_2$ was added to $MgSO_4$ in about equal proportions, the plants exhibited about 160 times the tolerance for the latter salt that they did in solutions of $MgSO_4$ alone. They found, further, that the antagonism between $CaCl_2$ and $MgCl_2$, though not so great (increasing the tolerance about 40 times), was nevertheless very marked, and where $CaSO_4$ replaced $CaCl_2$ the antagonism was very much greater between Ca and Mg than in either of the cases above cited.

I wish to cite only one more case, which emphasizes by strong contrast the exceptional results obtained above in experiments with *B. subtilis;* that is, the results of highly ingenious experiments on rabbits and a monkey by MELTZER and AUER (**16**) showing the antagonistic effect of calcium on the inhibitory effect of magnesium. As a

typical instance may be cited experiment 1 of their series, in which about 13^{cc} of an $m/1$ solution of $MgCl_2$ was injected subcutaneously. Less than half an hour later there was produced general anaesthesia, with all the attending symptoms. When 2^{cc} of a solution of $m/8$ $CaCl_2$ was injected into the ear vein the rabbit was again breathing normally, and when 8^{cc} had been given the animal sat up and appeared entirely recovered, except for a stiffness in the hind legs.

In these experiments, some of which were even more striking than the one cited, MELTZER and AUER employed, besides the chlorids of Ca and Mg, the acetate and nitrate of the former, and the acetate, nitrate, and sulfate of the latter. The same strong antagonism was noted in all cases.

TABLE VIII

ALL QUANTITIES GIVEN REFER TO CUBIC CENTIMETERS OF 0.35 m SOLUTIONS

Culture solution	Corresponding points on curve	Milligrams of N formed as NH_3
100 $MgCl_2$	K	4.76
100 $MgCl_2$ } 5 $CaCl_2$ }	J	4.48
100 $MgCl_2$ } 10 $CaCl_2$ }	I	4.20
100 $MgCl_2$ } 25 $CaCl_2$ }	H	3.78
100 $MgCl_2$ } 50 $CaCl_2$ }	G	3.64
100 $MgCl_2$ } 100 $CaCl_2$ }	F	3.22
50 $MgCl_2$ } 100 $CaCl_2$ }	E	3.08
25 $MgCl_2$ } 100 $CaCl_2$ }	D	3.29
10 $MgCl_2$ } 100 $CaCl_2$ }	C	3.43
5 $MgCl_2$ } 100 $CaCl_2$ }	B	3.57
100 $CaCl_2$	A	3.78

In addition to the confirmation of the results stated above in experiments with the same material, one series was also carried out with a culture of *B. subtilis* obtained from New Jersey, and with salt solutions made up from a different grade of chemically pure salt.

As can be seen from the following table and also from *fig. 4*, the results fully confirm those above given; and though the absolute amounts are different, the results are relatively the same.

It may be of interest to note here that *B. subtilis* from a 24-hour peptone agar slope was examined in hanging drops of molecular solutions of magnesium chlorid and calcium chlorid, and the organisms showed no perceptible ill effects from the action of the solution. The ciliary movements appeared normal even after 24 hours in the hanging drop. It was noticed, however, that there was little or no division during the 24 hours and it is likely that the calcium and magnesium salts exercise their toxic effects, partly at least, by inhibiting reproduction, since the ciliary movements seemed to go on without interruption. These remarks, however, are based on too meager experimental evidence to be anything else than conjecture at present, but they serve to indicate a field of most interesting research.

Though they are not analogous to the lack of antagonism between Ca and Mg shown above, it is interesting to note two cases on record, in which the addition of one salt to another made a combination more toxic than either. One case is that cited above from OSTWALD'S experiments on the freshwater Gammarus, in which a combination of $MgCl_2$ and NaCl in solution was more toxic to that animal than either of these in solution alone. The other case is that noted in the experiments of KRÖNIG and PAUL (2), who found that the value of mercuric sulfate, acetate, and nitrate as disinfectants was enhanced by the addition of small amounts of the chlorids of the alkalies (K and Na); but, on the other hand, that the addition of the same chlorids to $HgCl_2$ reduced considerably the disinfecting powers of the latter.

The first instance is not analogous to the results of the writer, because one of the salts used was different, and the experiment was carried out under conditions so totally different that the value of a comparison is doubtful. In the second instance, as KRÖNIG and PAUL themselves suggest in the same article, the increase of toxicity is not necessarily owing to a lack of antagonism between the two salts, but rather to the formation of complex double salts of mercury, which are characteristic of that element, and therefore this again cannot be compared with the lack of antagonism between Ca and Mg above noted.

SERIES VIII. SODIUM CHLORID *vs.* POTASSIUM CHLORID

This series was carried out to determine the antagonistic action between KCl and NaCl and was arranged in the same manner as the foregoing series. The ammonia determinations gave the following results:

TABLE IX

ALL QUANTITIES GIVEN REFER TO CUBIC CENTIMETERS OF 0.35 *m* SOLUTIONS

Culture solution	Corresponding points on curve	Milligrams of N formed as NH_3
100 NaCl	K	10.99
100 NaCl, 5 KCl	J	11.62
100 NaCl, 10 KCl	I	10.50
100 NaCl, 25 KCl	H	10.08
100 NaCl, 50 KCl	G	9.66
100 NaCl, 100 KCl	F	11.62
50 NaCl, 100 KCl	E	17.57
25 NaCl, 100 KCl	D	14.42
10 NaCl, 100 KCl	C	13.44
5 NaCl, 100 KCl	B	11.41
100 KCl	A	10.57

That the like valences of the two salts employed do not prevent antagonistic action in the case of *B. subtilis* (as may have been surmised from results in the last series) can be seen from the curve in *fig. 5*. Here again, there is a marked resemblance between the effect of this combination of metallic salts on *B. subtilis* and on animals and higher plants. LILLIE, for example, showed (3) that the ciliary activity of the larvae of Arenicola, which was inhibited in solutions of either KCl or NaCl alone, went on normally when solutions of the two at the same concentrations were mixed in the proportion of 20 parts NaCl to 8 parts KCl.

LOEB (9) and OSTWALD (22) also found, in working on a marine

and a freshwater Gammarus respectively, that a distinct antagonism exists between NaCl and KCl. Among plants we find that the work of OSTERHOUT (**20**) also shows some antagonism between KCl and NaCl, and here again it is significant to note that there are two maxima.

The curve in *fig. 5* shows an unusually gradual rise and decline.

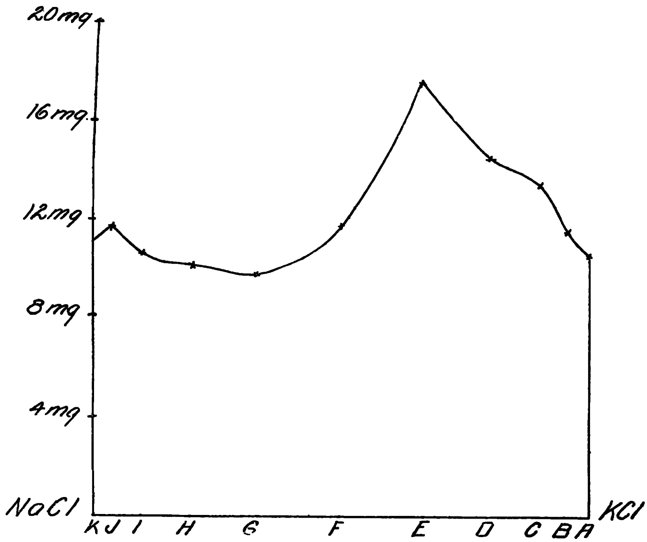

FIG. 5.—Antagonism curve, NaCl *vs.* KCl. The ordinates at K represent the ammonia nitrogen in milligrams formed in a pure NaCl solution. The ordinate at A represents the amount of ammonia nitrogen formed in a pure KCl solution, and the ordinates at the intermediate points represent the amounts formed in various combinations of the two salts as indicated by the corresponding letters in table IX.

This is characteristic of the Na and K chlorids used singly, where they become gradually more and more toxic with the increase of concentration. It is consequently in strong contrast to the sharp decline of the calcium and magnesium curves, especially that of the former. In this curve, as above noted, there are two maximal points, one at the combination of 100^{cc} of NaCl + 5^{cc} KCl, and another at the combination of 100^{cc} KCl + 50^{cc} NaCl. In three confirmatory series on this experiment, the maximal points were in each case obtained at the same combinations.

Discussion

Briefly reviewing the results with *B. subtilis* above given, we note their significance from the scientific as well as the practical standpoint. Of the four chlorids tested singly, NaCl is the only one stimulating ammonification at the concentrations employed. It is not unlikely, however, that KCl has a similar effect at a slightly lower concentration.

$CaCl_2$ is the most toxic of the chlorids used. In this feature, *B. subtilis* appears to resemble animals, for which calcium is very toxic, and not plants; since for plants, with which bacteria are now classed, calcium is the least toxic of the four chlorids. This fact may have a bearing on the future classification of bacteria.

The strong antagonism exhibited in some of the combinations of salts employed speaks eloquently for the fact that balanced solutions are as necessary for the optimal development of bacteria and allied forms as for the higher plants and animals, a fact which has been denied in a recent publication of LOEW and ASO (13). This is of the greatest practical significance when applied to soil bacteria, and especially those of alkali soils, in which, owing to the excessive amount of one or more salts, the bacterial activity is inhibited, and consequently plant food is not made available to the higher plants.

Because the salts experimented with are all found in our soils in larger or smaller amounts, and in some soils are present in excess, it is a matter of practical importance to apply the results of researches on the physiology of plants and bacteria to improve such alkali lands. There is no doubt in my mind that when we have learned to coordinate the results of researches on plants and soil bacteria, and to apply them in the field, we shall have at our command a method for the control and profitable cultivation of alkali lands, of which so many thousands of acres are merely vast wastes at the present time.

Lastly, the results of these experiments are significant because they open up an unexplored field of bacterial physiology, in which further researches will teach us much.

Summary

1. Each of the four chlorids ($CaCl_2$, $MgCl_2$, KCl, NaCl) is toxic for *B. subtilis*, in the order given, the first being the most toxic and

the fourth the least. This is quite different from the results with higher plants, where magnesium is the most toxic and calcium the least.

2. A marked antagonism exists between Ca and K, Mg and Na, K and Na.

3. No antagonism exists between Mg and Ca, but the toxic effect of each is increased by addition of the other to it. This is just the opposite of what has hitherto been found for plants.

In conclusion, the writer desires to express his indebtedness to Prof. W. J. V. OSTERHOUT, at whose instance these researches were begun, for helpful suggestions and kindly criticism throughout the course of the investigations.

LABORATORY OF SOIL BACTERIOLOGY
UNIVERSITY OF CALIFORNIA

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